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Review

Allosteric modulation of zinc speciation by fatty acids☆☆

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ABSTRACT

Background: Serum albumin is the major protein component of blood plasma and is responsible for the circulatory transport of a range of small molecules that include fatty acids, hormones, metal ions and drugs. Studies examining the ligand-binding properties of albumin make up a large proportion of the literature. However, many of these studies do not address the fact that albumin carries multiple ligands (including metal ions) simultaneously *in vivo*. Thus the binding of a particular ligand may influence both the affinity and dynamics of albumin interactions with another.
Scope of review: Here we review the Zn^{2+} and fatty acid transport properties of albumin and highlight an important interplay that exists between them. Also the impact of this dynamic interaction upon the distribution of plasma Zn^{2+}, its effect upon cellular Zn^{2+} uptake and its importance in the diagnosis of myocardial ischemia are considered.
Major conclusions: We previously identified the major binding site for Zn^{2+} on albumin. Furthermore, we revealed that Zn^{2+}-binding at this site and fatty acid-binding at the FA2 site are interdependent. This suggests that the binding of fatty acids to albumin may serve as an allosteric switch to modulate Zn^{2+}-binding to albumin in blood plasma.
General significance: Fatty acid levels in the blood are dynamic and chronic elevation of plasma fatty acid levels is associated with some metabolic disorders such as cardiovascular disease and diabetes. Since the binding of Zn^{2+} to albumin is important for the control of circulatory/cellular Zn^{2+} dynamics, this relationship is likely to have important physiological and pathological implications. This article is part of a Special Issue entitled Serum Albumin.

1. Introduction

Serum albumin is the most abundant protein in the circulatory system (constituting around 60% of total plasma protein) and is one of the most extensively studied. It is a single chain protein of ~66 kD and is made up of three homologous domains (I–III) each composed of two subdomains (A and B) [1]. Typically, albumin is present in the blood at a concentration of ~600 μM and contributes 80% to colloid osmotic blood pressure [2]. It is also thought to be responsible for the maintenance of blood pH [3]. However, albumin's most notable feature is its ability to bind reversibly and transport a vast array of small molecules, such as fatty acids, metal ions, toxic metabolites, hormones and drugs [2,4].

Collectively, ligand-binding studies constitute a large part of the albumin literature. However, the vast majority of these studies focus only on binary systems (*i.e.* between albumin and a particular small molecule). Although such studies provide important information relating to the chemistry of the apoprotein, albumin carries multiple ligands simultaneously *in vivo*. It is therefore essential that ternary systems are also examined so that we may fully understand the relationships that link the transport of different physiologically important small molecules by serum albumin. In this article, we highlight recent work demonstrating the interplay between the Zn^{2+} and fatty acid transport properties of albumin. The physiological and medical relevance of this dynamic interaction are discussed.

2. Zinc in plasma and role of albumin in zinc transport

Zinc was first discovered to be an essential nutrient for animals in the early 1930s in a study on the effect of dietary zinc intake on the growth and survival of mice and rats [5]. However it was not until the

Abbreviations: ACB, albumin-cobalt-binding; ATCUN, amino-terminal copper and nickel binding; BSA, bovine serum albumin; EXAFS, extended X-ray absorption spectroscopy; FA1–7, fatty acid-binding sites 1–7; HRG, histidine-rich glycoprotein; HRR, histidine-rich region; HSA, human serum albumin; IMA, ischemia-modified albumin; ITC, isothermal titration calorimetry; MI, myocardial ischemia; mol. eq., molar equivalents; TCA, tricarboxylic acid

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1960s that the importance of zinc for human health was fully realized [6]. A number of studies aimed at defining the interactions between proteins and Zn^{2+} in human blood plasma and serum have been conducted since [7–14], and there is now general agreement that serum albumin is the major Zn^{2+} transporter in mammalian plasma [15].

A typical reference value for the concentration of total zinc in normal adult human blood plasma is 16.6 ± 6.2 μM [16]. The plasma zinc pool is influenced significantly by dietary intake, and a range of other factors; for example, decreased plasma levels are encountered during infection and inflammation, as part of the acute phase response, but also after meals. The total turnover rate of plasma Zn^{2+} is considerable, with the total amount of zinc in plasma being replaced ~150 times/day [17].

A common approach to assess zinc speciation in plasma involves fractionation into high- and low-molecular weight fractions, *e.g.* by ultrafiltration [18]. More recently, zinc-responsive fluorescent dyes have been used to quantify total and labile zinc in plasma [19]. Measurements with the dye ZnAF-2 allowed a free Zn^{2+} concentration around 1–3 nM to be inferred and confirmed that most, but not all, of the Zn^{2+} binding capacity in plasma resides in the high-molecular weight fraction. Between 75 and 90% of total plasma zinc is bound to human serum albumin (HSA), and this fraction makes up the bulk of the exchangeable plasma zinc pool [11,13]. Less than 1% of total plasma zinc is reported to be associated with the low molecular weight fraction [18], which is thought to contain mainly zinc complexed by the amino acids histidine and cysteine [11,20]. The remainder of plasma zinc (*ca.* 10–20%) is tightly bound to α2-macroglobulin [21], making up the majority of non-exchangeable zinc in plasma, and retinol-binding protein (*ca.* 2%) [11].

The interaction between albumin and Zn^{2+} is therefore of particular importance within the blood. Studies on perfused rat intestine reveal that albumin is responsible for the transport of newly-absorbed Zn^{2+} to the liver [22]. Also albumin has been shown to facilitate the uptake of Zn^{2+} into endothelial cells [23] and erythrocytes [24].

Zinc binding to albumin has previously been probed by $^{111}Cd^{2+}$ and $^{113}Cd^{2+}$ NMR spectroscopy where two broad peaks with equal integrals were observed: one at 110–150 ppm (site A) and the second at 25–30 ppm (site B) [25–27]. The addition of 1 equivalent of Zn^{2+} suppressed the peak corresponding to site A, but left site B unaffected, which led to the conclusion that site A had a strong preference for zinc [25–27]. Recently, this high affinity zinc binding site has been located, modeled and characterized using site-directed mutagenesis, multinuclear NMR, and Zn k-edge EXAFS spectroscopy [28,29] (Fig. 1a–c). The site is essentially a 5-coordinate interdomain site involving residues His67 and Asn99 from domain I and His247 and Asp249 from domain II, plus one non-protein ligand (thought to be water). A sixth weaker coordination site is occupied by the backbone carbonyl oxygen of His247 (Fig. 1d).

Interestingly, Asp248 in domain II of BSA (corresponding to Asp249 in HSA) has been identified as also forming part of a Ca^{2+} binding site, suggesting that there may be some interplay between these two metal ions [30]. The binding of Zn^{2+} to albumin has recently been shown to affect Mn^{2+} binding [31]. In this study it was concluded that the high-affinity Zn^{2+} site corresponds to a secondary Mn^{2+} site. The anticancer drug cisplatin can also prevent Zn^{2+}-binding at this site through formation of a crosslink between the two histidine side-chains (His67 and His247) [32].

Besides helping with the identification of the major zinc binding site and the contributing residues, mutant recombinant human albumins can modulate the affinity of HSA for Zn^{2+}, as established by equilibrium dialysis experiments [29]. Replacement of the weakly-binding side-chain of Asn99 with a stronger-binding Asp or His ligand increases the Zn^{2+} affinity by at least an order of magnitude, whereas mutation of His67 to the non-coordinating Ala residue decreases the affinity. The corresponding EXAFS studies confirmed that both Asn99 mutants bind zinc in a similar 5(+1)-ligand site, whereas the data for the His67Ala mutant yielded an EXAFS fit with one ligand less (Fig. 1b). Analysis of 2D ^{1}H TOCSY NMR spectra for the mutants allowed the identification of the cross-peaks corresponding to His67 and His247 for wild-type HSA, which enabled monitoring of the effects of different binding partners (metals, fatty acids) on this site (see below).

3. Fatty acids in plasma and role of albumin in their transport

The so-called free (to indicate that they are non-esterified) short- and medium-chain fatty acids are absorbed into the blood following dietary intake [33]. However, plasma free fatty acids are dominated by long-chain (mainly C16 and C18) fatty acids, and these are primarily mobilized from triglyceride stores in adipose tissues *via* enzyme hydrolysis (lipolysis), which is tightly controlled [34]. They are then released into the circulation where they are transported by albumin (Fig. 2) [35]. Transport of fatty acids is characterized by rapid turnover, with a circulatory half-life of 2–4 min [36]. The precise mechanism by which albumin facilitates the uptake of fatty acids to cells/tissues is not known. However, the existence of specific albumin receptors that may aid in this process has been proposed [37,38]. The early work by Dole and Gordon explored the function and importance of fatty acids in the general metabolic process [39–41]. Work, particularly by Gordon, showed the origin of plasma fatty acids from adipose tissue and their utilization by the myocardium and liver. Fatty acids are the primary source of energy for many body tissues such as resting skeletal muscle, renal cortex, liver and myocardium [42]. The basal plasma concentration of fatty acid is approximately 250–500 μM [43], most of which comes from abdominal subcutaneous fat reserve and only a small fraction originates from intra-abdominal fat tissues [44,45]. When there is an increase for fuel demand by various body tissues, there is also an increase in adipose tissue lipolysis. This in turn, increases the systemic bioavailability of fatty acids [42].

Chronic elevated plasma fatty acid levels are associated with various disorders such as cancer, diabetes and obesity [46] and are a symptom of analbuminemia, a deficiency of plasma HSA [47]. Data published by others show that the level of plasma fatty acids increases in obesity largely due to enlargement of adipose tissue as well as a delay in their clearance [48]. Furthermore, elevated levels of plasma fatty acids inhibit the effects of insulin on lipolysis to cause further increase in the plasma fatty acids [49]. Pregnancy has been cited as a causative reason for elevated plasma fatty acid level. It has been reported that early in pregnancy, maternal fat is deposited while later in pregnancy lipolytic hormones cause fat break-down which subsequently leads to a higher level of fatty acids in plasma. This leads to a peripheral insulin resistance and a switch to fat oxidation from carbohydrate, hence maximizing the access of glucose to the fetus for development [50]. It has also been reported that the level of plasma fatty acids is age/sex-dependent. For boys the level of fatty acids in plasma decreases prior to puberty, whereas in girls, plasma fatty acid levels do not decrease with age [51].

Fatty acid-binding to albumin has been extensively studied by a range of biophysical techniques including dialysis [52], ^{13}C NMR [53,54] and X-ray crystallography [55–57]. Under physiological conditions it is thought that 0.1–6.0 molar equivalents (mol. eq.) of fatty acid can be bound to albumin (K_d values for binding of various long-chain fatty acids to HSA range between 1.5 and 90 nM [2]), >2 mol. eq. will bind only under conditions where plasma fatty acid levels are elevated [58]. Crystallographic studies have identified at least seven specific fatty acid binding sites on human albumin (FA1–7), which are asymmetrically distributed across the three domains [55,56,59]. Three of these sites are thought to be of high affinity (FA2, 4 and 5) [54,60], with two further sites of medium affinity (FA1 and 3). These sites (Fig. 2a) all entail defined salt bridges between the negatively charged fatty acid head-groups and positively charged protein side-chains as well as other hydrogen bonding interactions which stabilize the interaction between the protein and the fatty acid ligand [55,56]. Two further, still weaker sites (FA6 and 7) were also observed in X-ray crystal structures, but these are located at the protein surface, with relatively poorly defined interactions.

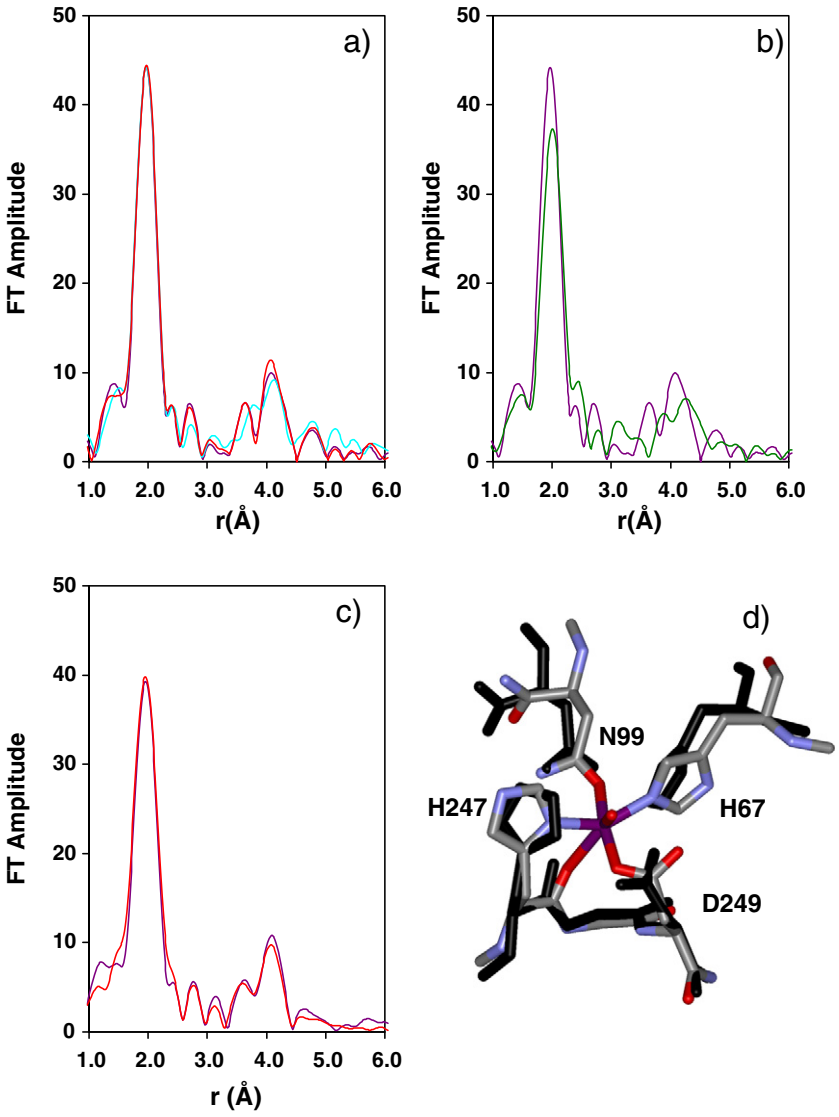

Fig. 1. Zn k-edge EXAFS data [29] for wild-type and mutant Zn-albumin complexes. (a) Comparison of three different preparations of wild-type rHA. Purple: rHA + 1 mol. eq. Zn^{2+}. Red: rHA + 1 mol. eq. Zn^{2+} + 1 mol. eq. Cd^{2+}. Cyan: rHA + 1 mol. eq. Zn^{2+} + 1 mol. eq. Cd^{2+} + 1 mol. eq. Cu^{2+}. In the latter two preparations, Cd^{2+} is thought to occupy preferentially site B, and Cu^{2+} the N-terminal ATCUN site. The excellent agreement in the first shell (intense peak at ca. 2 Å) suggests that in each preparation, Zn^{2+} was bound to the same site. (b) Comparison of EXAFS spectra for wild-type (purple) and H67A mutant (green) HSA. The H67A mutation clearly perturbs zinc binding to albumin. (c) Overlay of experimental (purple) and fitted (red) data. The fit corresponds to the model shown in (d). (d) Overlay of residues forming the zinc site on albumin in published X-ray structure of unliganded albumin (black; PDB ID: 1AO6 [124]), and in EXAFS-refined model (Zn ion shown in purple, oxygen in red, nitrogen in blue).

4. Albumin mediates crosstalk between zinc and fatty acids

Following identification of the Zn^{2+}-binding site on albumin it was noted from available structural data that this site is essentially pre-formed, but only in X-ray structures of fatty acid-free albumin. The key fatty acid binding site responsible for these structural differences is FA2 (Fig. 2b). It is one of the highest affinity sites and becomes significantly occupied at fatty acid:albumin ratios as low as 1:1 [54]. It is also one of the most enclosed sites with its almost linear binding pocket formed by two half pockets from subdomains IA, and IIA and IIB [59]. In fatty acid-free albumin, these two half-sites are however 10 Å apart, and hence fatty acid binding to this site requires a considerable conformational change that involves the rotation of domain I with respect to domain II [55,56]. It is the same conformational change that also profoundly changes the distance between the two pairs of zinc-binding residues in domains I and II, allosterically influencing the binding of Zn^{2+}; essentially, there is no longer a pre-formed zinc binding site in this region once FA2 is occupied (Fig. 2b). This is the case for all reported X-ray crystal structures of fatty acid-bound albumin, in which chain lengths of bound fatty acids range from C10 to C22 [53,56,61,62]. The latter observation led to the hypothesis that zinc binding to site A and fatty acid binding to FA2 might be mutually exclusive, but it was unclear whether zinc would preclude fatty acid binding or *vice versa*.

Early experimental evidence for fatty acids perturbing metal binding came from ^{113}Cd NMR spectroscopy, where peak A was significantly broadened and of reduced intensity in non-defatted albumin samples isolated from plasma [27]. Subsequently it was demonstrated for recombinant Cd_2HSA that the presence of an 8-fold excess of octanoate (C8) also had a large perturbing effect on the ^{111}Cd peak corresponding to site A while site B was unaffected (Fig. 3a) [28]. Titrating octanoate into HSA also affected the Hε1 ^1H NMR resonances for His67 and His247, suggesting that octanoate binds in the vicinity of these residues (Fig. 3b) [63]. However, from these data it was also concluded that octanoate binding to FA2 did not abolish zinc binding to site A, since the resulting ^1H spectra of Zn–HSA in the presence and absence of octanoate were identical. Thus it was not clear whether HSA bound Zn^{2+} more strongly to site A than octanoate to FA2, leading to octanoate displacement, or whether simultaneous binding was possible. The latter

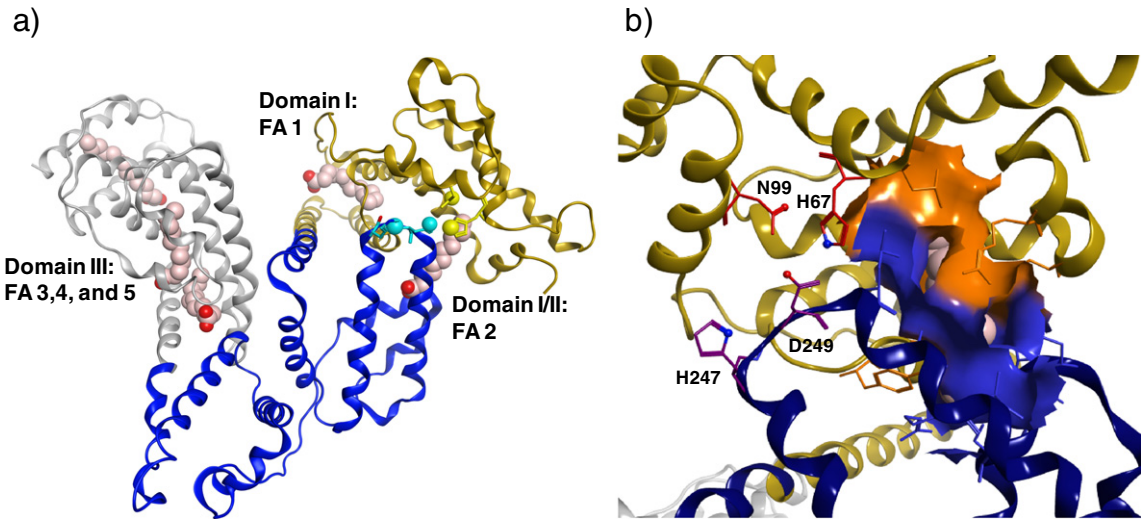

Fig. 2. (a) Location of the five major fatty acid binding sites of HSA (pink spheres; carboxyl oxygens in red), in relation to the residues forming the major zinc binding site (interacting atoms of N99 and H67 shown as yellow spheres, and those of H247 and D248 as cyan spheres). The backbone of the three domains is shown in yellow, blue and grey as labelled. (b) Fatty acid binding site FA2 and zinc site A residues. The fatty acid binding pocket formed by residues from both domain I (yellow/orange) and domain II (blue) is illustrated as a colored surface, with the carbons of the fatty acid molecule shown as pink space-filling spheres. Metal-binding nitrogen and oxygen atoms are picked out as spheres. To appreciate the effect of the fatty acid-induced conformational switch, compare the distance between the two His residues in Fig. 1d (*ca.* 4.3 Å) and here (>8 Å). Images 2a and b were generated using PDB ID:1BJ5 [55].

conclusion was reached by employing isothermal titration calorimetry (ITC) to study the interactive binding of Zn^{2+} and octanoate to bovine serum albumin (BSA); the binding curves for both ligands were identical irrespective of the presence or absence of the other ligand. Significantly, it had been previously reported that HSA in the presence of octanoate could not be crystallized in a form that was isomorphous to all other fatty acid-bound structures [56]; hence it is conceivable that octanoate does not elicit the same conformational change observed upon binding of longer chain fatty acids, as the C8 chain is too short to pin the two half-sites together. A structural model confirmed the plausibility of simultaneous binding of octanoate and Zn^{2+} [63]. Regarding the octanoate-induced disappearance of peak A in ^{111}Cd NMR spectra, it is possible that the binding of octanoate to one half pocket merely affects the dynamics of Cd^{2+} in site A, without necessarily displacing the metal.

In contrast, ITC experiments to study competition between Zn^{2+} and the C14 fatty acid myristate clearly demonstrated that these influenced the binding of each other to BSA (Fig. 4a and b). An increasing myristate concentration led to a significant decrease in the Zn^{2+}:BSA stoichiometry and overall affinity, whereas Zn^{2+} did not affect the binding stoichiometry of myristate, but rendered the binding reaction less exothermic. These observations indicated that the affinity for myristate (including that of site FA2) was higher than that for Zn^{2+} [63], consistent with literature data. The ITC experiments captured the occupation of two Zn^{2+} sites, and both of them were affected by the myristate concentration; at a 5:1 myristate:BSA ratio, virtually no Zn^{2+} binding could be detected under the experimental conditions used. An effect on a second metal site was also reflected in ^{111}Cd NMR spectra: like octanoate, a 5-fold molar excess of myristate also suppressed peak A in ^{111}Cd NMR spectra, but the peak for site B also disappeared [63]. This suggests that myristate binding could have an additional impact on site B, which is a likely candidate as a secondary site for Zn^{2+}. Structural characterization of site B is currently lacking, but the effect of fatty acid binding suggests that it may also be an interdomain site. Importantly, the ITC data revealed that the zinc binding capacity and affinity of BSA are affected by the addition of as little as 1 mol. eq. of myristate. Hence, fatty acids modulate the affinity of albumin toward Zn^{2+} at all physiological levels, not just in extreme conditions. Notably, an effect of the first equivalent is also consistent with the finding that FA2 is one of the three highest affinity sites [54].

The ITC competition experiments conclusively demonstrated that binding of zinc and long-chain fatty acids to albumin is interactive, as suggested by inspection of X-ray structures. Ultimately, this fatty acid "switch" mechanism could mean that albumin is a molecular link between the levels and distribution of fatty acid (which is reflective of energy status) and zinc in plasma and adjacent tissues. The potential biological consequences of such a shift in zinc distribution will be discussed in the next section.

5. Implications for fatty acid-induced modulation of metal ion binding and transport

Interestingly, numerous links between Zn^{2+} homeostasis/signaling and energy metabolism exist [64,65]. Zinc affects concentration and activities of the hormones insulin [66], glucagon [67], and leptin [68,69]. Notably, insulin is required for adequate HSA synthesis; diabetics have a decreased rate of albumin synthesis, which in-turn may influence molecular transport of fatty acids by albumin [4]. Leptin is produced in adipocytes and regulates energy intake and expenditure, and it is thought that through this link, Zn^{2+} status exerts a direct impact on eating behavior and appetite [70]. In obesity, hyperleptinemia is coupled with hypozincemia (measured as plasma zinc) [71]. Zinc also impacts on the activity of the hormone adiponectin, which is also secreted by adipocytes, is involved in regulating fatty acid oxidation, and also plays a role in insulin resistance [72]. Both insulin and adiponectin directly interact with Zn^{2+} ions. Their oligomerization and hence activity is dependent on the presence of Zn^{2+}. Lipogenesis (*i.e.* fatty acid synthesis and esterification) in adipocytes is enhanced by Zn^{2+} *in vitro* [71], and during the formation of fat tissue, Zn^{2+} is actively transported into adipocytes [73]. An elevation in intracellular Zn^{2+} may directly impact on leptin signaling *via* inhibiting protein tyrosine phosphatase 1B, which in turn inhibits phosphoinositide 3-kinase, a key enzyme in the leptin (and insulin) signaling pathways [74].

Although only ~2% of circulating albumin molecules carry a zinc ion, the modulation of their mutual affinity may have significant consequences. Broadly two possibilities need to be considered: (i) the reduced affinity for fatty acid-loaded albumin may lead to an altered plasma zinc speciation, which may include binding to alternative proteins, and (ii) a shift in plasma zinc speciation may result in different zinc uptake by endothelial cells, with further downstream effects. In addition, the

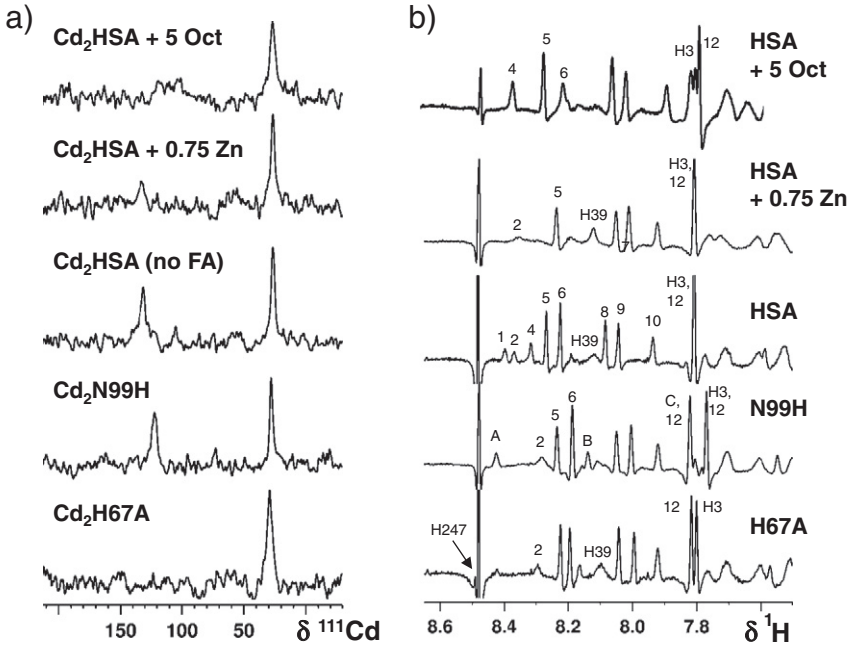

Fig. 3. NMR studies of wild-type and mutant albumins. (a) ^{111}Cd NMR spectra (1.5 mM Cd_2HSA, 50 mM Tris, 50 mM NaCl, pH 7.4). The middle spectrum shows pure Cd_2HSA. Both Zn^{2+} and octanoate peturb Cd^{2+} binding to the peak at ca. 130 ppm corresponding to site A. Mutations of zinc-binding residues either shift the peak (N99H mutant) or perturb it (H67A). (b) Metal and fatty acid binding monitored by resolution-enhanced ^{1}H NMR spectroscopy. The sharp peaks correspond to His Hε1 protons. Peaks 1 and 4 have been assigned to residues H67 and H247 [29]. Both are affected by Zn^{2+}, octanoate, and mutations of H67 or N99.

reduction in metal affinity may also be the molecular basis of a current clinical assay, the ACB test for myocardial ischemia. In the following, we will discuss these hypotheses (summarized in Table 1) in the context of clinical or physiological observations.

5.1. Re-distribution of zinc within plasma: Activation of histidine-rich glycoprotein

Histidine-rich glycoprotein (HRG) is a 75 kDa protein present in plasma at micromolar concentrations (~1.5 μM). Structurally, HRG consists of two cystatin derived N-terminal domains (named N1 and N2), a central histidine rich region (HRR) flanked at either end by a proline rich region and a C-terminal domain [75]. The distinct domains, held together by an arrangement of six disulfide bonds, enable HRG to bind to a multitude of molecules including, plasminogen, fibrinogen, thrombospondin, IgG, heparin/heparan sulfate, heme, Fcγ receptors and phospholipids [76–84]. The diversity of ligands recognized by HRG is related to its role as a regulator of numerous physiological processes. One such process is the coagulation of blood.

HRG binds to the anticoagulant, heparin, with a strong affinity to inhibit the formation of a heparin–antithrombin III complex. The heparin–antithrombin III complex regulates activated coagulants such as thrombin in a negative manner [85]. The HRG–heparin interaction therefore has a pro-coagulatory effect, and the formation of this complex is

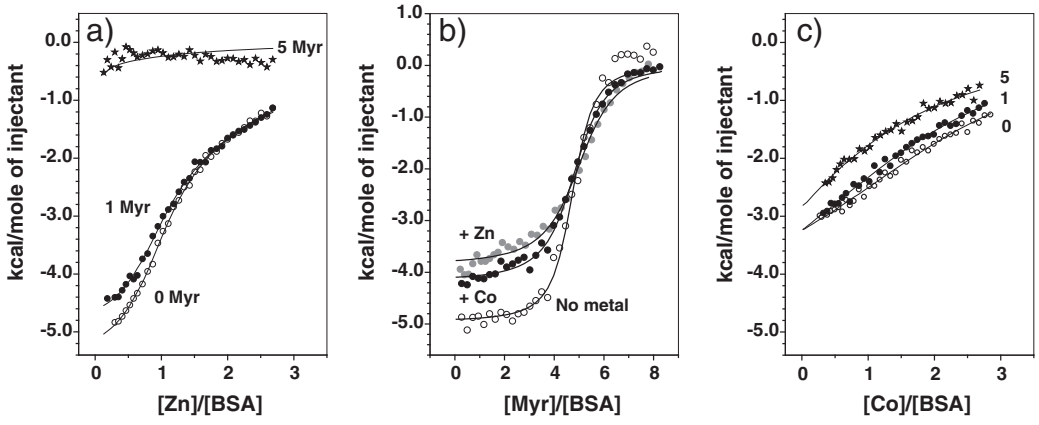

Fig. 4. Studying crosstalk between metal and fatty acid binding to albumin by ITC. (a) Titration of BSA (25 μM; 50 mM Tris, 50 mM NaCl, pH 7.2, 298 K) with Zn^{2+} (333 μM) in the presence and absence of myristate. Under the given conditions, zinc binding to two sites is captured, with apparent binding constants of log $K' = 5.67$ and 4.15. Correction for the influence of pH and the weak Zn^{2+} ligand Tris gave a stoichiometric log $K = 7.0$ for the strongest site. Myristate at both high and low concentrations affects zinc-binding to BSA, to the point that no binding can be detected in the presence of 5 mol. eq. of myristate under the given conditions. (b) Titration of BSA (12.5 μM, in H_2O, 298 K) with myristate in the presence and absence of Zn^{2+} or Co^{2+}. While fatty acid binding stoichiometry was not affected, there was a reduction in exothermicity, as it is thought that the binding of FA2 requires the removal of metal ions in site A and/or B. (c) Co^{2+} binding to BSA was studied under conditions identical to those used for Zn^{2+} binding to facilitate direct comparisons. Note that the scales in A and C are also identical. Clearly, Co^{2+} binding is significantly weaker than Zn^{2+} binding, in agreement with literature [125,126], but an effect of myristate is still evident. In contrast to the effect on Zn^{2+} binding, 5 mol. eq. of myristate did not completely abolish Co^{2+} binding. This may indicate that either site B is less affected than site A, and/or that Co^{2+} can compensate for the loss of site A (and B) by binding to the N-terminal ATCUN motif. Figure reprinted from [127] with permission from Elsevier.

enhanced in the presence of Zn^{2+} [83]. An interesting feature of HRG is the high prevalence of histidine and proline residues, each accounting for approximately 13% of the amino acid sequence. The HRR bears an extraordinary repeating amino acid sequence of Gly-His-His-Pro-His, developing a chain of histidine residues which can be implicated in metal binding.

Early work by Morgan showed that HRG is able to bind Cu^{2+}, Zn^{2+}, Hg^{2+}, Cd^{2+}, Ni^{2+} and Co^{2+} [86,87]. Unsurprisingly, Zn^{2+} binding was found to be pH dependent; because of proton competition for the imidazole nitrogens, less binding is observed at acidic pH. Also, it was observed that rabbit HRG is able to compete with albumin with respect to Zn^{2+} binding. This is intriguing since most dissociation constants reported for zinc complexes of albumin are lower than those for HRG (*e.g.* $K_{d(HSA)} = 30–100$ nM *cf.* $K_{d(HRG)} = 1–4$ μM). As previously pointed out, under normal conditions, the majority of exchangeable Zn^{2+} is bound to albumin, not least because of its much higher concentration [88]. Nevertheless, despite the well-known uncertainties in measurements of metal affinity data which are also greatly affected by pH and ionic strength, it is clear that the Zn^{2+} affinities of the two proteins are relatively close together.

It has been reported that human HRG is capable of binding up to 10 mol. eq. of Zn^{2+}, effectively multiplying the HRG binding site concentration [89]. Hence, it is possible that relatively small perturbations may lead to non-negligible shifts in Zn^{2+} distribution between these two proteins. In addition, it has been proposed that the plasma concentration of "free" Zn^{2+} may reach the levels required for Zn^{2+}-dependent HRG complex formation during thrombosis following release of Zn^{2+} from platelet-derived α-granules at the site of the thrombus [83]. It is thought that this process may aid in the spatial and temporal regulation of blood clotting [90]. Whether albumin plays a role in subsequent removal of this HRG-bound Zn^{2+} is not known.

In some individuals where plasma fatty acid levels are elevated, it is possible that release of exchangeable Zn^{2+} (by fatty acid-binding to albumin) may enhance the formation of HRG–heparin complexes. There is some indirect evidence that this may occur. For example blood analbuminemia is associated with hypercoagulability [91]. Also dietary studies have shown that some fatty acids can increase platelet aggregation and fibrinolysis [92,93]. Thus it is feasible that this mechanism may contribute to the pro-thrombotic pathologies associated with disorders such as cancer, diabetes and obesity [46]. It is therefore interesting to examine the relationship between fatty acid-binding to albumin, the subsequent release of Zn^{2+} and possible increased complexation of zinc by HRG.

5.2. Re-distribution of zinc between plasma and other compartments

Various pieces of evidence point towards albumin exerting an influence on Zn^{2+} uptake into cells: (i) For fibroblasts, albumin has been shown to affect uptake, essentially by acting as an extracellular zinc-binding ligand that buffers the free Zn^{2+} concentration [94]. (ii) In contrast, for endothelial cells, it was shown that albumin-bound Zn^{2+} can be taken up by endocytosis, and that albumin carrying Zn^{2+} has a higher affinity for the cells than the apoprotein [24]. (iii) For red blood cells, a supportive role of albumin has been suggested [25]. Hence, irrespective of whether albumin participates directly or indirectly in Zn^{2+} uptake by cells, it can be anticipated that a modulation of its Zn^{2+} affinity will impact on cellular Zn^{2+} uptake, although of course other mechanisms for regulating cellular uptake, including the upregulation of expression of membrane-bound zinc transporters, are also clearly important [95].

As previously mentioned, although plasma zinc levels are relatively constant, there are a number of conditions in which these levels are decreased [17]. Due to the multiple links between zinc and lipid (and energy) metabolism, it is difficult to dissect causes and consequences, but in order to gauge the plausibility of a contribution of the albumin fatty acid switch in Zn^{2+} redistribution, it is of interest to explore whether there are any physiological or clinical conditions in which both elevated free fatty acid levels and depressed plasma zinc levels may be encountered. Conditions relating broadly to energy metabolism and for which depressions in plasma zinc levels have been reported include strenuous aerobic exercise [74], obesity [96], diabetes [97,98], and cardiovascular disease. In all of these conditions, plasma fatty acid levels are also elevated [99].

Regarding cardiovascular disease, a finer distinction is necessary. All conditions that lead to a lower energy consumption by the heart could (in theory) lead to at least temporary elevations of free fatty acids, as these provide a large proportion of the energy consumed by the heart, in particular the myocardium. Therefore, it is possible that the lower plasma zinc levels observed in acute myocardial infarction [100–103], and chronic heart failure [104], have a direct correlation to high levels of free fatty acids. Serum zinc levels are known to fall sharply within few hours after the onset of myocardial infarction, with lowest values observed around 2–3 days, after which they return slowly to normal values [100]. Strikingly, a reduced metal-binding capacity of plasmas was found in patients with myocardial ischemia (MI), and this observation was developed into a clinical test — the albumin-cobalt-binding (ACB) assay [105], discussed in the next section.

5.3. Is ischemia-modified albumin normal albumin in the presence of elevated fatty acids?

MI describes the lack of oxygen supply to the heart muscle and is a precursor for acute myocardial infarction. Based on the hypothesis that MI might elicit modifications to circulating albumin that affect its metal binding capability, Bar-Or and colleagues developed the albumin-cobalt-binding (ACB) assay [105]. It was demonstrated that serum (or plasma) from patients with ischemia displays a significantly lower capacity for the binding of Co^{2+}, which can be rapidly measured colorimetrically through the formation of an unidentified brown adduct formed by reacting excess Co^{2+} with dithiothreitol. The ACB assay is the only available clinical test for the early and rapid assessment of MI [106], and has a good negative prognostic value: a normal reading allows MI and hence imminent hearts attack to be excluded [107–109]. However, the specificity of the test is low [110]; abnormal readings indicating low cobalt binding capacity have not only been found in the case of ischemia, but a plethora of other conditions, including obesity, diabetes [111], metabolic syndrome, fatty liver [112], cancer, infections, renal disease, pre-eclampsia, stroke, and even sustained exercise [113,114]. The utility of the test has therefore been questioned.

The biomarker that is thought to be detected by the ACB assay has been termed "ischemia-modified albumin" (IMA), with the underlying hypothesis that ischemia causes modifications to the ATCUN (amino-terminal copper and nickel binding) motif, such as N-terminal

Table 1
Risk factors, mechanism and potential medical implications associated with fatty acid-induced disruption of the primary zinc-binding site on albumin.

Risk factors	Mechanism	Medical implications
Elevated fatty acid levels are associated with disorders such as diabetes, obesity, cardiovascular disease, fatty liver disease and cancer.	Increased fatty acid binding to albumin leads to allosteric disruption of zinc binding site.	Displaced zinc binds to HRG, enhancing HRG–heparin complex formation leading to increased blood clotting (thrombosis). Altered cellular zinc uptake leading to hypozincemia. Cobalt also binds to albumin at the primary zinc site. Elevated plasma fatty acids levels (as associated with myocardial ischemia) lead to a positive ACB test.

truncation, a well-known albumin modification that occurs with storage over long time [115], or oxidation. Hence, Co^{2+}-binding to peptides as models for the N-terminus has been studied [116], but crucially, modifications to the N-terminus in albumin from ischemic patients (and with reduced cobalt-binding affinity) could not be demonstrated [117,118]. The molecular mechanism of reduced Co^{2+}-binding has therefore remained elusive. Several studies suggested that the N-terminus was not the major site for Co^{2+}-binding and that sites A and B were more relevant [119,120]. It should be emphasized that the N-terminus can in principle bind Co^{2+} and Zn^{2+} [121], but while the square planar environment generated by the ATCUN motif is well suited to the low-spin d^8 and d^9 metal ions Ni^{2+} and Cu^{2+}, respectively, this site is not as preferable for the d^7 ion Co^{2+} (and even less attractive to the d^{10} ion Zn^{2+}).

Faller had highlighted the possibility that if site A played a significant role in Co^{2+} binding, then fatty acids may influence Co^{2+} binding, too [119]. Meanwhile, the direct effect of elevated fatty acids in plasma on the cobalt-binding capacity has been demonstrated [112], and a "plausible but not causative" correlation between plasma fatty acids and IMA was reported [122], but still the molecular mechanism had not been revealed. Our previous Zn-related studies indicated an effect of fatty acids also on site B [63], which had been identified as the major site for Co^{2+} binding [120]. Based on these *in vitro* studies, our own work and published clinical observations, we hypothesized that the elusive biomarker "IMA" may simply correspond to normal albumin in the presence of elevated fatty acids. We tested this idea by studying the competitive binding of Co^{2+} and myristate by ITC, in analogy to the ITC studies described above for Zn/myristate competition, and demonstrated that Co^{2+} binding was indeed affected by the presence of myristate (Fig. 4b and c) [123]. Binding of Co^{2+} was less affected than binding of Zn^{2+} in the presence of 5 mol. eq. of myristate, such that 1.3 mol. eq. of Co^{2+} was still able to bind. This smaller effect was ascribed to differences between the affinities of the three sites for Zn^{2+} and Co^{2+}, and the different effects of fatty acids on these three sites. Site A is most impaired, followed by site B (based on ^{111}Cd NMR and ITC data), with no major negative impact anticipated on the N-terminus, based on structural considerations. Hence, since site A is the primary site for Zn^{2+}, but only a secondary site for Co^{2+}, complete elimination of site A has a massive impact on Zn^{2+} binding, but a lesser one for Co^{2+}, and this can also be compensated by alternative binding of Co^{2+} to the N-terminus. A mock ACB test using physiologically relevant concentrations of BSA and 0–5 mol. eq. of myristate also demonstrated a correlation between the absorbance readings of the test and fatty acid concentration [123].

This demonstrated, albeit *in vitro*, that elevated plasma fatty acids can directly influence the ACB assay. Furthermore, since this "modification" is non-covalent, it explains the rapid (within hours) return to normal IMA levels once the ischemic event is over. Given the long half-live of albumin, any covalent modifications are unlikely to be cleared as quickly, whereas clearance of plasma fatty acid occurs on a comparable time scale [36]. However, it is important to note that the suggested molecular mechanism does not exclude other mechanisms that influence albumin cobalt-binding such as reduced pH as a consequence of acidosis.

6. Conclusions

Although albumin chemistry is of undoubted importance and has been widely studied, many features of its behavior and its physiological consequences are not yet fully understood. We have attempted to illustrate this for Zn^{2+} and fatty acid binding. X-ray crystal structures are tremendously helpful but do not provide the full picture. For example, there is as yet no X-ray structure of albumin with Zn^{2+} bound. Solution structures may differ from solid-state X-ray crystal structures and in solution there can be dynamic exchange reactions depending on the relative affinity constants (which themselves are likely to be dependent on the solution conditions (pH, ionic strength *etc.*). Also both Zn^{2+} and fatty acid binding are difficult to study and in solution it is necessary to make indirect inferences as to the structural changes they induce. It seems clear though that the interactive binding of Zn^{2+} and longer chain fatty acids has potential physiological and clinical consequences. Further work is necessary to fully understand its importance. It is hoped that the work highlighted here will motivate others to examine interactive binding of multiple ligands to albumin so that we may fully understand the relationships that govern their transport in plasma.

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